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MICROBIAL CONVERSION OF PLANT STEROL (B, SITOSTEROLS) INTO IMPORTANT STEROID ANDROGENS USING NEW LOCALLY ISOLATED BACTERIA

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ABSTRACT

Microbial biotransformation of phytosterol to produce androstanedione (AD and androstadiendione (ADD) is a valuable interest reaction since. it considers to be steroid hormones intermediates. The current study deals with the bioconversion of β, sitosterol to produce (AD) and (ADD) using new bacterial isolate. Some physiological and biochemical factors such as inoculums size, inoculums age, pH, temperature and substrate concentrations were investigated. The results showed that the maximum yield of AD (42.3%) was obtained by using 24 h inoculum age and 2 ml/ 100 ml inoculum size; pH8; temperature at 30ºc; as well as 15 mg/100ml substrate concentration.

KEYWORDS: 4-androstene-3, 17-dione (AD), androst-1,4-diene-3,17-dione (ADD), Phytosterols, Microbial biotransformation.

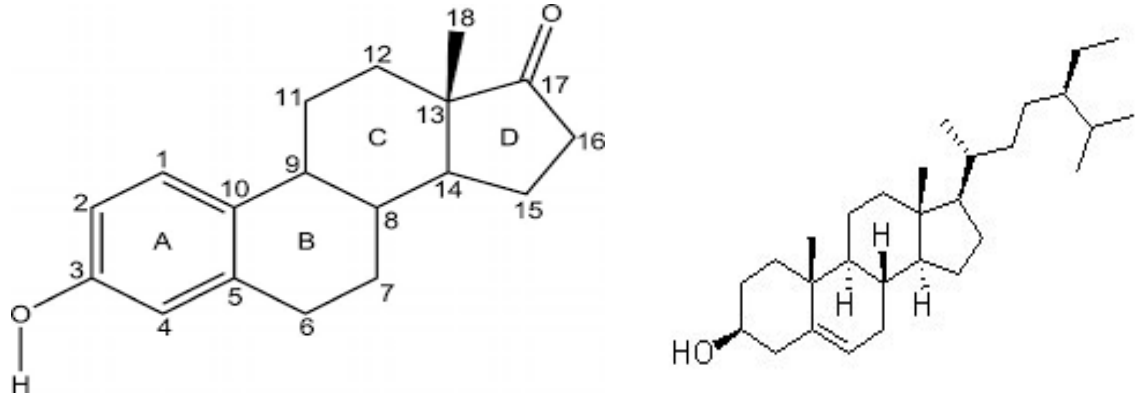
1. INTRODUCTION

Steroids are terpenoid lipids of specific structure that contain the nucleus of four cycloalkane rings. Androstenedione (AD) is a natural steroid which belongs to the 17-ketosteroid family. Itis produced by the adrenal cortex and gonads. In the body, cholesterol leads to the formation of steroidal hormones.

Phytosterols gained an increasing importance as raw materials for the synthesis of steroidal drugs such as pregnenolone, boldenone, androstenedione and androstadienedione. Phytosterols are thoroughly widespread in plants and are similar to cholesterol in terms of

physiological functions and structure.[1] Phytosterols differ from cholesterol by having a methyl or ethyl group at C-24 (**Figure1**). Phytosterols participate in essential cellular processes since, they modulate permeability and fluidity of membranes. In addition, they are precursors forthe synthesis of steroid hormones and are involved in plant defense mechanisms. β-Sitosterol campesterol, and stigmasterol are the main phytosterols found in plants.[1]

Both AD and ADD are compound specifically used as a precursor for the majority ofpharmaceutically active steroids such as testosterone, estradiol, ethinylestradiol, testolactone, progesterone, cortisone, cortisol, prednisone and prednisolone.[2]



A **B**

Figure 1: Structure of (A) androstendione and (B) β sitosterols.

However, the low solubility of β-sitosterol in aqueous system limits the sitosterol available for biotransformation. The cell wall structure of *Mycobacterium* sp., normally used as biocatalyst, also impedes the transport of sitosterol into the cytosol. Mycobacterial cell wall consists of peptidoglycan, arabinogalactan and mycolic acids, which form the mycobacterial cell wall skeleton'.[3]

The aim of the present investigation focuses on the mechanisms used for enhanced important pharmaceutical intermediates namely, AD and ADD which play an important role in the production of many steroidal hormones under optimized conditions by using the isolated bacterial strain which successfully isolated from soil from Egypt, which could transform β-sitosterol under optimized conditions.

MATERIALS AND METHODS

Materials

Isolation of microorganisms

The used bacteria was isolated from the soil and biochemical identification carried out as well as identified by PCR technique.

Chemicals

The authentic steroids (AD, ADD) and β, sitosterol, were provided by sigma company USA.

Solvent systems

1. **Benzene:** Ethyl acetate: Acetone (4: 1: 1 V/V)
2. **Benzene:** Cyclo hexane: (1:1 V/V)

Colour Reagents: Libermann – Burchard's reagent 5 ml sulphuric acid, 5 ml acetic anhydride, 45 ml absolute alcohol

Methods

Preparation of inoculums

Materials

The inoculum was prepared by using Erlenmeyer flasks 250 ml containing 50 ml of the flowing medium (g/L) glucose 10 K_2HPO4 0.75, KH_2PO4 3, $(NH4)_2SO41$, $MgSO4 .7H_2O$ 1, 8 hydroxyqunioline 0.8) the pH was initially adjusted to 6.5.[3] The sterile medium was inoculate using a spore suspension and incubated at 200 rpm, 30°C, 24 hr.

Transformation process

250 ml Erlenmeyer flask containing 100ml of the above medium was inoculated by 2ml from the previously prepared inoculums.[4] It incubated for 24h then induced by 1 mg β, sitosterol/100 ml medium, after which 10 mg of β, sitosterol was added to each flask and the bioconversion process was continued to 72 h at 180 rpm and30± 1°C. Due to the limited solubility of the sterol of the nutrient solution, it was dissolved in the minimal volume of ethyl alcohol.

Separation and Analysis of the transformation

At the end of the transformation period the product were analyzed according to the method described by **Salam,** *et al*.[4] The contents of each flask were extracted with twice its volume

of chloroform, and the solvent layer was separated and evaporate under vacuum to give semi solid residue" test material". This material undergo qualitative and quantitative analysis.

Qualitative

The qualitative analysis was carried out by using thin chromatography technique(TLC) according to the methods described by **Sallam *et al.*,**[4]

Quantitative determination of AD and ADD profile by gas chromatography (GC)

Gas chromatography (Hewlett Packard, 6890) equipped with a flame ionization detector was used. Oven with initial temperature 180$C^0$and maximum 300C^0 at initial time 0 min, equilibration time 3 min and run time 40 min and a capillary column (model number; HP 19091Z-413 HP1-methyl siloxane) with a maximum temperature 325C^0, nominal length: 30 m, nominal diameter: 320.00 mm, nominal film thickness 0.25 mm. The mode was constant flow 2 ml/min, inlet pressure 14.83 Pa; average velocity 41 cm/s and back inlet mode was splitless at initial temperature. Detector temperature 295 C^0 as the hydrogen flow was 30 ml/min and air flow was 350 ml/min. Helium was the carrier gas at a flow rate of 30 ml/min . Identification of the components, were carried out by comparing retention time Rt to these of standards AD (25.45 min, ADD 23.40 min campsterol 34.18 min, stigmasterol 35.13 min and β, sitosterol 36.68 min.[5]

RESULTS

Factors affecting the optimization production (FAOP) of both AD and ADD

1-Inoculums age effects on the bioconversion of β, sitosterol

Different inoculums ages (12,24,48, 72, 96 h) were investigated. The results given in Fig. (2). clearly indicated that the selection of inoculums age of the used inoculums was an important factor in the AD productivity, the inoculums age of the 24 hours was accompanied with the best AD output 5.44 mg/100ml. After that the decrease in AD output was recorded 1.66 by using an inoculums age of 96 h inoculums age have effect on AD.

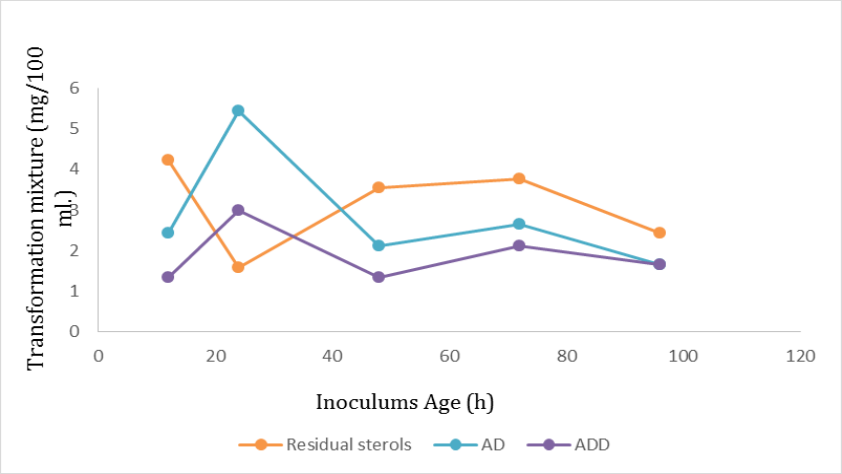


Fig. 1: Effect of different inoculums age on the bioconversion of β, sitosterol.

2- different inoculums size on the bioconversion of β, sitosterol

Different inoculums sizes ranges from (0.5 – 5) ml/ (100 ml were studied).The results presented in fig. (2) revealed that the inoculums size of tested bacteria play important role in the bioconversion of β, sitosterol and produce AD and ADD .The highest production of AD and ADD given (5.33 and 3.36 mg /100 ml) respectively at (2 ml/ 100 ml) inoculum size.

At the higher inoculums sizes (3,4,5 ml /100 ml) the bioconversion products were decreased. Therefore 2ml/100 ml it will be selected to continue the subsequent experiments.

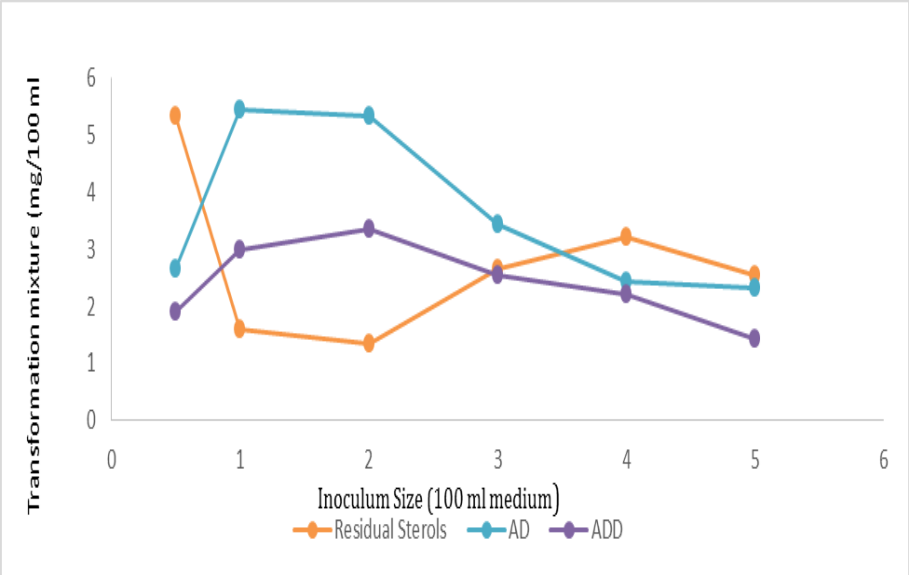


Fig. 2: Effect of different inoculums size on the bioconversion of β, sitosterol.

3-pH relations

It is well known that among the different physiological factors affecting the transformation of β, sitosterol process is the pH of the fermentation medium. Accordingly, the bioconversion of was investigated at different pH values of the used fermentation basal medium.

From the results presented in Fig. (3) it could be detected that the bio conversion efficiency was markedly affected by the initial pH value of the fermentation medium. The best yield of AD (6.78 mg/100ml) was recorded at pH 8. On the other hand, the pH changes towards the more acidic side produced remarkable decrease inthe outputs of AD and ADD. The variation in the hydrogen ion concentration in the fermentation medium wasreflected by remarkable changes in AD and ADD productivity.

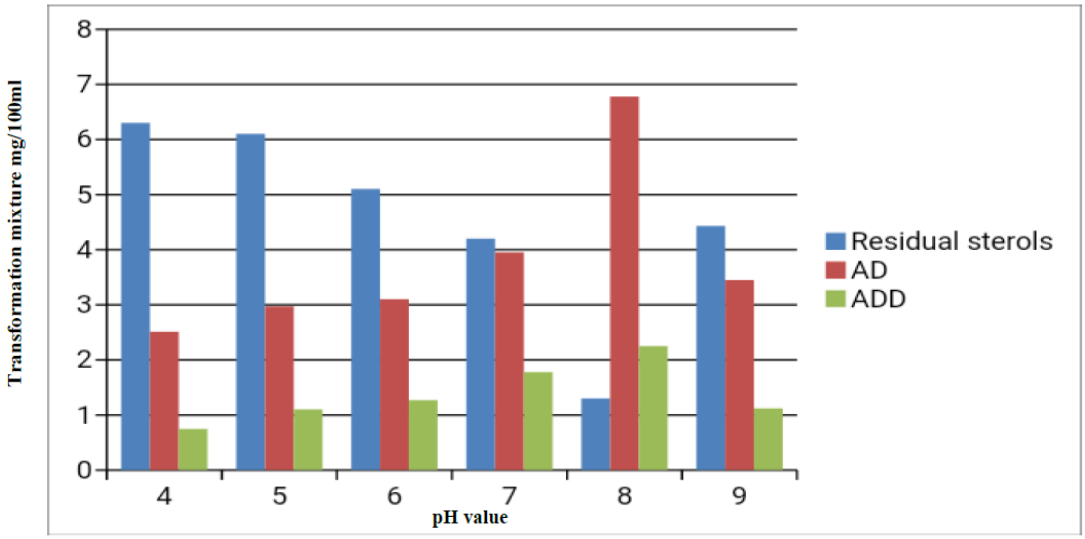


Fig. 3: Effect of different pH value of the fermentation medium o the bioconversion of β, sitosterol.

4-Different temperature effects on the bioconversion of β, sitosterol

This was approached by assaying the transformation at different temperature intervals (20, 25, 30, 35 and 40) ^{0}C. It was intended to follow up the rate of transformation of the AD and ADD using experimental organisms to select the proper temperature at which the maximal activities of side chain degradation reaction take place. It is clearly evident from the results given in Fig(4) that the capacity of experimental organism to produce AD and ADD is considerably affected by the temperature at which the fermentation process is terminated. Obviously, a definite temperature should be permitted to attain a relatively high AD out puts.

Thus maximal yield (6.21) mg/100ml for AD was estimated at 30^{0}C. At 20 and 35 ^{0}C lower out outs of AD were obtained (1.78 and 2.22) mg/100ml respectively.

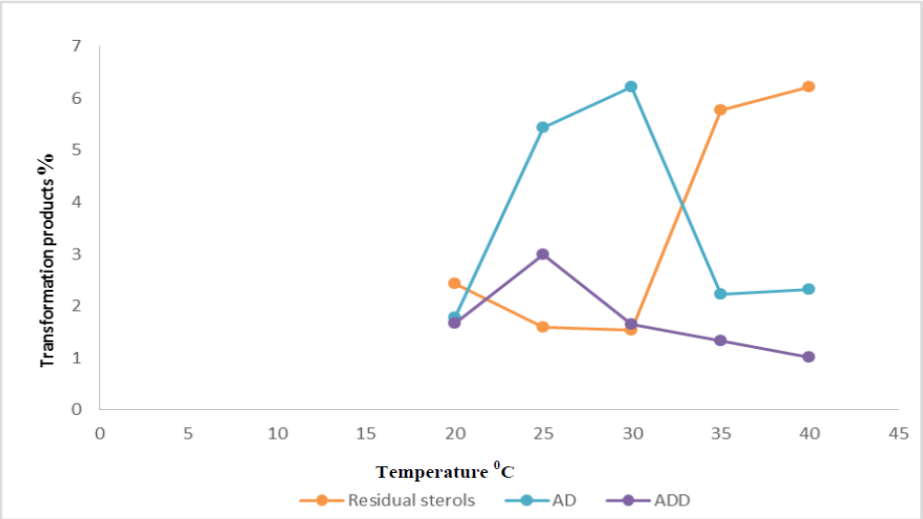


Fig. 4: Effect of different temperature on the bioconversion of β, sitosterol.

5-Substrate concentration suitability on the bioconversion process

The present investigation extended to study the effect of different substrate concentrations (5, 10, 15, 20, 25 mg/100ml medium) on the bioconversion efficiency. The results presented in Fig. (5) showed that the best bioconversion activity was (46.03%) was obtained at substrate oncentration 15g/100ml medium. On the other hand, at the higher substrate concentration remarkable reduced levels of the transformation products were obtained due to substrate toxicity.

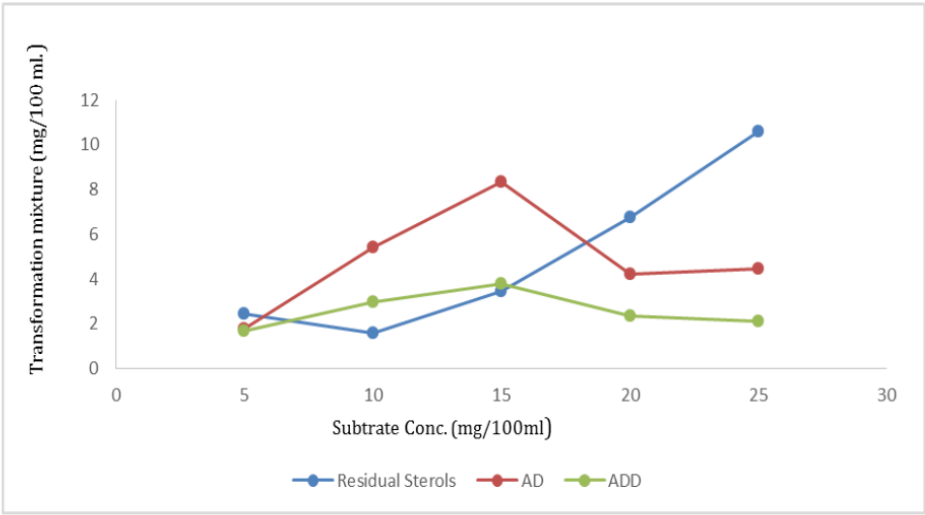


Fig. 5: Effect of different substrate concentration on the bioconversion of β, sitosterol.

6-Phylogenic analysis of the isolate

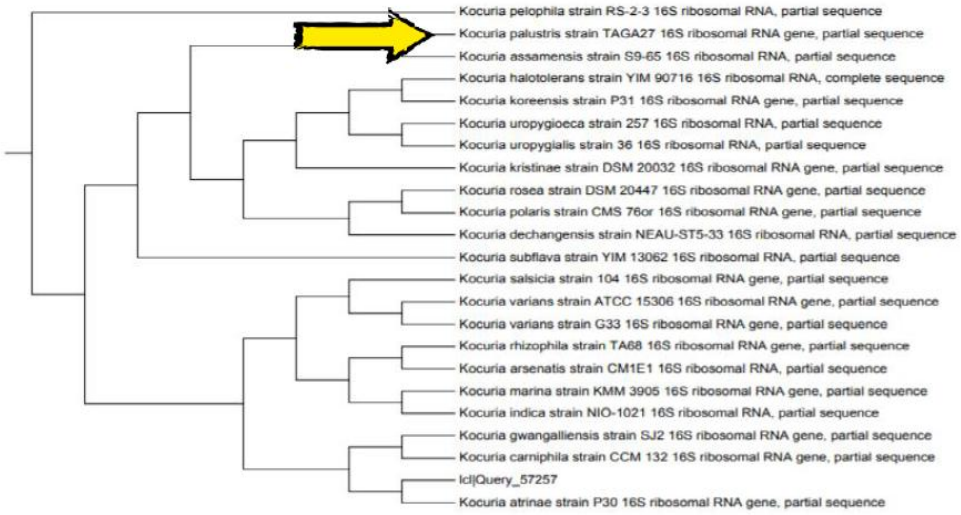


Fig. 6: Phylogenetic-tree showing detailed genotype of *Kocuria* palustiria strain P30 16S ribosomal RNA gene, partial sequence.

DISCUSSION

Sterols of plant origin (phytosterols) are used as mainraw materials in steroid pharmaceutical industry.[6] Oxidative degradation of the aliphatic side chain of phytosterols with selected wild-type, or engineered strains of actinobacteria, mostly, saprotrophic mycobacteria is a well-established method for production of3-oxo-androstane steroids such as androst-4-ene-3,17-dione (AD) andandrosta-1,4-diene-3,17-dione (ADD) which are the key intermediates for the preparation of valuable steroid pharmaceuticals and produced in huge amounts.[7,8]

In turn, structural modifications of AD/ADD, and in particular, regio- and stereospecific hydroxylation allows obtaining the molecules of high biological activity, The study of factors influencing the biotransformation of steriod is very much essential in any bioprocess development. Generally a higher productivity has been achieved by pH, temperature, inoculums size, inoculums age and substrate concentration optimization.

Considering the previous results the best yield of AD (6.78 mg/100ml) was recorded at pH 8.Optimum pH 8 was reported by **Goswami *et al*.**[9] for production of AD (D) from 17-ketosteroids by *Arthrobacter*. In addition, optimal pH between 6 and 8 for steroid biotransformation had referred by several reports as[10] they reported optimal pH at 7. **Abd-Elsalam *et al*[11]** reported optimum pH 6.5 for production of androstenedione (AD) and

androstadienedione (ADD) from sunflower oil sterols by *Fusarium solan* where, they reported that the highest boldenone yield 53.6% obtained at pH 8 that was the optimum pH.

The fermentation temperature showed maximal yield (6.21) mg/100ml for AD was estimated at 30ºC. This result seemed to be in a good agreement with most of reports dealing with this topic as**.**[12,13]

The best bioconversion activity was (80.6%) was obtained at substrate concentration 15g/100ml medium, at the higher substrate concentration a remarkable reduced levels of the transformation products were obtained due to substrate toxicity, these results agree with[14] also reported the similar adverse effect at high concentration of the substrate. This result was in accordance with the discovery by **Smith** *et al***.**[15] reported that the effect of different concentrations of soysterols on the bioconversion by *M. fortuitum* subsp. *fortuitum NCIM 5239* indicated a steady decrease in the conversion to AD with increasing concentration of soysterols. Besides results obtained by **Abd-Alla.,**[16] The study of different initial phytosterol concentrations from 5 to 30 g/L on the conversion efficiency and ADD production were investigated, higher phytosterol conversion efficiency and biomass was observed.[17] While, with the increasing phytosterol concentration, the phytosterol conversion efficiency and the biomass decreased dramatically.

It is clearly evident from the data given in table () the inoculums age of the 24 hours was accompanied with the best AD output 5.44 mg/100ml in spite of negative effect of the dry weight by increasing inoculums age up to 96 hours. This results agree with **Shao**, *et a*l.,[17] who used 24h culture medium of *Nocardioides simplex* as inoculums for AD production in synthetic medium.

On the other hand**,** compared between two inoculation strategies for the bioconversion of wood sterols by Mycobacterium sp. used fermented broth with 72 h inoculums age. In this connection[18] used 48hr inoculums age to inoculate the synthetic media used to investigate the biotransformation of the phytosterols mixture of the filter cake mud to produce AD and ADD.

The inoculum size of 2% (v/v) was the most proper one and leads to the highest production of AD and ADD given (5.33 and 3.36 mg /100 ml) respectively.

In this connection Shao et al., (2015) who studied improve the androst-1,4-diene-3,17-dione (ADD) production from phytosterol by Mycobacterium *neoaurum JC*-12 and used 10% (v/v) seed culture as inoculum.[19] founded that the optimal conditions for the production of androstenedione by microbial assay were determined by orthogonal test at inoculum volume 15% (v/v) Shtrantaikova *et al.*,[19] reported that 8% (v/v) inoculum increase the productivity of boldenone by direct conversion of phytosterol .

CONCLUSION

Conclusions In this study, *Kocuria palusteria* strains which could utilize phytosterols wastes by biotransformation process used for enhanced the production of AD and ADD under optimized conditions by apply factors such as inoculums size, inoculums age, pH, temperature and substrate concentrations. The results showed that the maximum yield of AD (42.31%) was obtained by using 2 ml/ 100 ml inoculum size; pH 8; temperature at 30ºc; 24 h inoculum age and 15 mg/100ml substrate concentration.

REFERENCES

1. Chudasama, K.and Thaker, V Genome sequence of ochrobacterium anthropic strain Sl BG 007 aplant pathogen and potential xenobiotic degradation bacterium.Genomics data , 2017; 11: 116-117.
2. Malaviya. A and Gomes, J, Androstendione production by biotransformation of phtyosterols. Bioresource technol, 2008; 99(5): 6725-6737.
3. Galan, BUhin L. Qarria, Fernandez, G Martinez, Bahill, K Fuent J., Barrondoand Garein J. Mycobaterium snegnitisis a suitable cell factory for the production of steroids synthesis. Microbial biotechnol, 2017; 10, 1: 38-150.
4. Sallam, L. Osman, M., Hamdy, A. and Zaghlol, G. Microbial transformation of phytosterol mixture from rice bran oil unsaponofiable matter by selected bacteria. Wourld journal of microbiology and biotechnology, 2008; 24, 1643-1656.
5. Abd-Elsalam, I., Salam, L, and salah Optimization of sugar cane phytosterols bioconversionusing *Arthrobacter rubellus*. Journal od appl. Sc. Res, 2010; (1): 1334-1339.
6. Donnova, M., Dovhynva, Kwan and Han Microbial conversion of sterols containg soybean oil production waste. J. chemical tecniol. Biotechnol, 2005; 80, 1: 55-60.
7. Wang, W, and Yu. Lpreparation of hydrocortisone from cortexolone 21 acetateby immobilized Absidia orchids. Enz. Microb. tech, 2011; 22(5): 368-373.

8. Fernandez- cabenzic, L. GatinB., and Goren J. New insight on steroid biotechnology . Forentiers in microbiology, 2018; 9: 958.
9. Gaswani, P, Singh, Hi and Barauh, J. Factors limiting the microbial conversion of steroids to. 17, ketosteroids in the presence of metal chelate inhibitors. Folia microbiologia, 1984; 29(3): 209-216.
10. Abd-Elsalam, I., El-Refai, and Aboulftouh. Facterial desigen for the production of some steroidal hormones from sun flower oil sterols by Fusareium solani. J. appl. Sci, 2007; 3(10): 921-925.
11. Smith, M Singh, S. and Singh R. Microbial biotransformation a process for chemical aleternations. J. bac. Myco. open acess, 2017; 4(2): 85.
12. Kumar, R. Dahiya, A. Singh, D. and Nigm, P (hang, R. Bao, Z, Yaam, C. Can, H. Sch, J. Sum, J. and Zhang B. iotransformation of phytosterols to androst 1, 4 diee 3, 17 dione by Mycobacterium spp. ZFZ expersing 3, ketosteroid dehydrogenase. Catalysis, 2020; 10(6): 663.
13. Xu, XGao, X, Feng, J. Wang., Xand Wei, C Inflence of temp. on nucleus degredation of 4- androstene3,17dione in phytosterol biotransformation by Mycobaterium sp. Ltters in Appl. Microbiol, 2015; 6(1): 63-68.
14. Gar, X Reng, I. Hun, Q. and WangX. Investigation of factors affecting biotransformation of phytostrols to 9 hydroxy androst-4 ene 3, 17 dione based on HPBCD resting cells, reaction system. Biocatalysis an biotransformation, 2014; 32(5): 343-347.
15. Abd-alla, A. Microbial conversion of sugar cane phytosterols by Fusarium solani.. Inter. J. research in Pharm and Biomed. Sci, 2013; 2(4): 14-21.
16. Shao, M, Zhang, X. Rao, Z., Xu, M Yang, Li and XuZ. Enhanced production of androst 1, 4 diene 3, 17 dione by Mycobacterium neounrum Jc-12 using three stage fermentationstratigy. PLOS, one, 2015; 10(9).
17. Zhang, X Peng, X, Su, Z., Chen, Q. Ruan, H and He, G. Optimization of biotransformationfrom phytosterols to androstendiione by a mutant Mycobacterium neonurum Z!MN.. J. of Zhejang Unver. Sc, 2013; 14(2): 132- 143.
18. Shtrantnikova, V, Schelknow M., Fokina, V. Pekov, V. Ivashina, and Donova, M. Genome –wide bioinformatics analysis of steroid metabolism associated gene in Nocardinodes simplex VEM. Current genetics, 2016; 62(3): 643-656.